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(54) Title: ENZYME–MEDIATED MODIFICATION OF FIBRIN FOR TISSUE ENGINEERING

Growth normalized against
unmodified fibrin
* p<0.05 vs. Fibrin
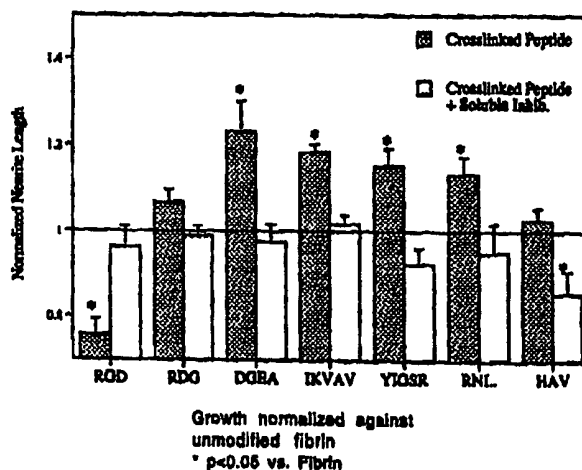
(57) Abstract

　　The invention provides fibrin–based, biocompatible materials useful in promoting cell growth, wound healing, and tissue regeneration. These materials are provided as part of several cell and tissue scaffolding structures that provide particular application for use in wound–healing and tissue regenerating. Methods for preparing these compositions and using them are also disclosed as part of the invention. A variety of peptides may be used in conjunction with the practice of the invention, in particular, the peptide IKVAV, and variants thereof. Generally, the compositions may be described as comprising a protein network (e.g., fibrin) and a peptide having an amino acid sequence that comprises a transglutaminase substrate domain (e.g., a factor XIIIa substrate domain) and a bioactive factor (e.g., a peptide or protein, such as a polypeptide growth factor), the peptide being covalently bound to the protein network. Other applications of the technology include their use on implantable devices (e.g., vascular graphs), tissue and cell scaffolding. Other applications include use in surgical adhesive or sealant, as well as in peripheral nerve regeneration and angiogenesis.

ENZYME-MEDIATED MODIFICATION OF
FIBRIN FOR TISSUE ENGINEERING

BACKGROUND OF THE RELATED ART

Fibrin is a natural gel with several biomedical applications. Fibrin gel has been used as a sealant because of its ability to bind to many tissues and its natural role in wound healing. Some specific applications include use as a sealant for vascular graft attachment, heart valve attachment, bone positioning in fractures and tendon repair (Sierra, D.H., *Journal of Biomaterials Applications*, 7:309-352, 1993). Additionally, these gels have been used as drug delivery devices, and for neuronal regeneration (Williams, *et al.*, *Journal of Comparative Neurobiology*, 264:284-290, 1987).

The process by which fibrinogen is polymerized into fibrin has also been characterized. Initially, a protease cleaves the dimeric fibrinogen molecule at the two symmetric sites. There are several possible proteases that can cleave fibrinogen, including thrombin, reptilase, and protease III, and each one severs the protein at a different site (Francis, *et al.*, *Blood Cells*, 19:291-307, 1993). Each of these cleavage sites have been located (FIG 1). Once the fibrinogen is cleaved, a self-polymerization step occurs in which the fibrinogen monomers come together and form a non-covalently crosslinked polymer gel (Sierra, 1993). A schematic representation of the fibrin polymer is shown in FIG 2. This self-assembly happens because binding sites become exposed after protease cleavage occurs. Once they are exposed, these binding sites in the center of the molecule can bind to other sites on the fibrinogen chains, these sites being present at the ends of the peptide chains (Stryer, L. *In Biochemistry*, W.H. Freeman & Company, NY, 1975). In this manner, a polymer network is formed. Factor XIIIa, a transglutaminase activated from factor XIII by thrombin proteolysis, may then covalently cross-link the polymer network. Other transglutaminases exist and may also be involved in covalent crosslinking and grafting to the fibrin network.

Once a crosslinked fibrin gel is formed, the subsequent degradation is tightly controlled. One of the key molecules in controlling the degradation of fibrin is α2-plasmin inhibitor (Aoki, N., *Progress in Cardiovascular Disease*, 21:267-286, 1979). This molecule acts by crosslinking to the α chain of fibrin through the action of factor XIIIa (Sakata, *et al.*,

5   *Journal of Clinical Investigation*, 65:290-297, 1980). By attaching itself to the gel, a high concentration of inhibitor can be localized to the gel. The inhibitor then acts by preventing the binding of plasminogen to fibrin (Aoki, *et al.*, *Thrombosis and Haemostasis*, 39:22-31, 1978) and inactivating plasmin (Aoki, 1979). The α-2 plasmin inhibitor contains a glutamine substrate. The exact sequence has been identified as NQEQVSPL (SEQ ID NO: 15), with

10   the first glutamine being the active amino acid for crosslinking.

The components required for making fibrin gels can be obtained in two ways. One method is to cryoprecipitate the fibrinogen from plasma. In this process, factor XIII precipitates with the fibrinogen, so it is already present. The proteases are purified from plasma using similar methods. Another technique is to make recombinant forms of these

15   proteins either in culture or with transgene animals. The advantage of this is that the purity is much higher, and the concentrations of each of these components can be controlled.

Cells interact with their environment through protein-protein, protein-oligosaccharide and protein-polysaccharide interactions at the cell surface. Extracellular matrix proteins provide a host of bioactive signals to the cell. This dense network is required to support the

20   cells, and many proteins in the matrix have been shown to control cell adhesion, spreading, migration and differentiation (Carey, *Annual Review of Physiology*, 53:161-177, 1991). Some of the specific proteins that have shown to be particularly active include laminin, vitronectin, fibronectin, fibrin, fibrinogen and collagen (Lander, *Journal of Trends in Neurological Science*, 12:189-195, 1989). Many studies of laminin have been conducted,

25   and it has been shown that laminin plays a vital role in the development and regeneration of nerves *in vivo* and nerve cells *in vitro* (Williams, *Neurochemical Research*, 12:851-869, 1987; Williams, *et al.*, 1993), as well as in angiogenesis.

Some of the specific sequences that directly interact with cellular receptors and cause either adhesion, spreading or signal transduction have been identified. This means that the

30   short active peptide sequences can be used instead of the entire protein for both *in vivo* and *in vitro* experiments. Laminin, a large multidomain protein (Martin, *Annual Review of Cellular Biology*, 3:57-85, 1987), has been shown to consist of three chains with several

receptor-binding domains. These receptor-binding domains include the YIGSR (SEQ ID NO: 1) sequence of the laminin B1 chain (Graf, et al., Cell, 48:989-996, 1987; Kleinman, et al., Archives of Biochemistry and Biophysics, 272:39-45, 1989; and Massia, et al., J. of Biol. Chem., 268:8053-8059, 1993), LRGDN (SEQ ID NO: 2) of the laminin A chain (Ignatius, et al., J. of Cell Biology, 111:709-720, 1990) and PDGSR (SEQ ID NO: 3) of the laminin B1 chain (Kleinman, et al., 1989). Several other recognition sequences for neuronal cells have also been identified. These include IKVAV (SEQ ID NO: 4) of the laminin A chain (Tashiro, et al., J. of Biol. Chem., 264:16174-16182, 1989) and the sequence RNIAEIIKDI (SEQ ID NO: 5) of the laminin B2 chain (Liesi, et al., FEBS Letters, 244:141-148, 1989). The receptors that bind to these specific sequences have also often been identified. A subset of cellular receptors that has shown to be responsible for much of the binding is the integrin superfamily (Rouslahti, E., J. of Clin. Investigation, 87:1-5, 1991). Integrins are protein heterodimers that consist of α and β subunits. Previous work has shown that the tripeptide RGD binds to several $\beta 1$ and $\beta 3$ integrins (Hynes, R.O., Cell, 69:1-25, 1992; Yamada, K.M., J. of Biol. Chem., 266:12809-12812, 1991), IKVAV(SEQ ID NO: 4) binds to a 110 kDa receptor (Tashiro, et al., J. of Biol. Chem., 264:16174-16182, 1989; Luckenbill-Edds, et al., Cell Tissue Research, 279:371-377, 1995), YIGSR (SEQ ID NO: 1) binds to a 67 kDa receptor (Graf, et al., 1987) and DGEA (SEQ ID NO: 6), a collagen sequence, binds to the $\alpha_2\beta_1$ integrin (Zutter & Santaro, Amer. J. of Pathology, 137:113-120, 1990). The receptor for the RNIAEIIKDI (SEQ ID NO: 5) sequence has not been reported.

Work has been done in crosslinking bioactive peptides to large carrier molecules and incorporating them within fibrin gels. By attaching the peptides to the large carrier polymers, the rate of diffusion out of the fibrin gel will be slowed down. In one series of experiments, polyacrylic acid was used as the carrier polymer and various sequences from laminin were covalently bound to them to confer neuroactivity (Herbert, C. in Chemical Engineering 146) to the gel. The stability of such a system was poor due to a lack of covalent or high affinity binding between the fibrin and the bioactive molecule.

Very little work has been done in incorporating peptide sequences and other bioactive factors into fibrin gels and even less has been done in covalently binding peptides directly to fibrin. However, a significant amount of energy has been spent on determining which proteins bind to fibrin via enzymatic activity and often determining the exact sequence which binds as well. The sequence for fibrin γ-chain crosslinking has been determined and the

exact site has been located as well (Doolittle, *et al.*, *Biochem. & Biophys. Res. Comm.*, 44:94-100, 1971). Factor XIIIa has also been shown to crosslink fibronectin to fibronectin (Barry & Mosher, *J. of Biol. Chem.*, 264:4179-4185, 1989), as well as fibronectin to fibrin itself (Okada, *et al.*, *J. of Biol. Chem.*, 260:1811-1820, 1985). This enzyme also crosslinks von

5   Willebrand factor (Hada, *et al.*, *Blood*, 68:95-101, 1986), as well as α-2 plasmin inhibitor (Tamaki & Aoki, *J. of Biol. Chem.*, 257:14767-14772, 1982), to fibrin. The specific sequence that binds from α-2 plasmin inhibitor has been isolated (Ichinose, *et al.*, *FEBS Letters*, 153:369-371, 1983) in addition to the number of possible binding sites on the fibrinogen molecule (Sobel & Gawinowicz, *J. of Biol. Chem.*, 271:19288-19297, 1996) for

10   α-2 plasmin inhibitor. Thus, many substrates for factor XIII exist, and a number of these have been identified in detail.

SUMMARY OF THE INVENTION

15   The present invention in a general and overall sense, provides unique fusion proteins and other factors, either synthetically or recombinantly, that contain both a transglutaminase domain such as a Factor XIII, a substrate domain and a bioactive factor, these peptides being covalently attached to a fibrin substrate having a three-dimensional structure capable of supporting cell growth.

20   In some embodiments of the present invention, bioactive properties found in extracellular matrix proteins and surface proteins are confined to a structurally favorable matrix that can readily be remodeled by cell-associated proteolytic activity. In some embodiments, the fibrin is gel matrix. A bioactive means is also included to facilitate the incorporation of an exogenous signal into the substrate. In addition to retaining the

25   bioactivity of the exogenous signal molecule, the overall structural characteristics of the fibrin gel is maintained.

         The invention in another aspect provides for a fibrin matrix comprising short peptides covalently crosslinked thereto, as well as bioactive factors. The fibrin matrix may be further defined as a fibrin gel. The matrix chosen is fibrin, since it provides a suitable three

30   dimensional structure for tissue growth and is the native matrix for tissue healing. It is anticipated that other, fibrin-like matrices may also be similarly prepared. The crosslinking was accomplished enzymatically by using the native Factor XIII to attach the exogenous

factors to the gels. In order to do this, a sequence that mimics a crosslinking site was incorporated into the peptide so that the enzyme recognized and crosslinked it into the matrix. Novel activity will be conferred to these fibrin gels by adding a peptide sequence, or other bioactive factor, which is attached to the crosslinking sequence. These materials may be

5   useful in the promotion of healing and tissue regeneration, in the creation of neovascular beds for cell transplantation and in numerous other aspects of tissue engineering. Hence, the invention in yet other aspects provides compositions created and adapted for these specific uses.

The following sequences are referenced throughout the Specification:

10

SEQ ID NO:	DESCRIPTION
SEQ ID NO: 1	YIGSR - Peptide that binds to a 67 kDa receptor
SEQ ID NO: 2	LRGDN - Peptide of the laminin A chain
SEQ ID NO: 3	PDGSR - Peptide of the laminin B1 chain
SEQ ID NO: 4	IKVAV - Peptide that binds to a 110 kDa receptor
SEQ ID NO: 5	RNIAEIIKDI - Peptide of the laminin B2 chain
SEQ ID NO: 6	DGEA - A collagen peptide that binds to the α_2, β_1 integrin
SEQ ID NO: 7	PRRARV - A sequence from fibronectin is also a heparin sulfate binding sequence
SEQ ID NO: 8	YRGD*TIGEGQQHHLGG* - A peptide with glutamine at the transglutaminase coupling site, an active RGD sequence and a dansylated amino acid, mimics the crosslinking site in the γ chain of fibrinogen
SEQ ID NO: 9	LRGD*GAKDV* - A peptide that mimics the lysine coupling site in the δ chain of fibrinogen with an active RGD sequence and a dansylated leucine added

SEQ ID NO: 10	LRGKKKKG - A peptide with a polylysine at a random coupling site attached to an active RGD and a dansylated lecine
SEQ ID NO: 11	L*NQEQVSPL*RGD -A peptide that mimics the crosslinking site in α 2-plasmin inhibitor with an active RGD added to the carboxy terminus and a dansylated leucine to the amino terminus
SEQ ID NO: 12	YRGD*TIGEGQQHHLGG* - A peptide with glutamine at the transglutaminase coupling site in the chain of fibrinogen
SEQ ID NO: 13	*GAKDV* - A peptide that mimics the lysine coupling site in the chain of fibrinogen
SEQ ID NO: 14	KKKK - A peptide with a polylysine at a random coupling site
SEQ ID NO: 15	*NQEQVSPL* - A peptide that mimics the crosslinking site in 2- plasmin inhibitor
SEQ ID NO: 16	*LNQEQVSPLG*YIGSR - A peptide that mimics the crosslinking site in α2-plasmin inhibitor with an active YIGSR added to the carboxy terminus and a dansylated leucine to the amino terminus
SEQ ID NO: 17	*LNQEQVSPL*DDGEAG - A peptide that mimics the crosslinking site in α2-plasmin inhibitor with an active DGEA
SEQ ID NO: 18	*LNQEQVSPLR*AHAVSE - A peptide that mimics the crosslinking site in α2-plasmin inhibitor with an active HAV added to the carboxy terminus and a dansylated leucine to the amino terminus
SEQ ID NO: 19	*LNQEQVSPR*DIKVAVDG - A peptide that mimics the crosslinking site in α2-plasmin inhibitor with an active IKVAV added to the carboxy terminus and a dansylated leucine to the amino terminus

SEQ ID NO: 20	*LNQEQVSPR*NIAEIIKDIR - A peptide that mimics the crosslinking site in α2-plasmin inhibitor with an active RNIAEIIKDI added to the carboxy terminus and a daysylated leucine to the amino terminus

In one aspect, the invention provides a composition that comprises a protein network and a peptide having an amino acid sequence that comprises a transglutaminase substrate

5   domain and a bioactive factor (e.g., peptide, protein, or fragment thereof) is provided. The peptide is covalently or at least substantially covalently bound to the protein network. In particular embodiments, the protein network is fibrin or a fibrin-like molecule. In other particular embodiments, the transglutaminase substrate domain is a factor XIII, a substrate domain. This factor XIII, a substrate domain may be further defined as comprising an amino

10   acid sequence SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, a fragment thereof, a combination thereof, or a bioactive fragment of said combination. Some embodiments may be defined as comprising a bioactive factor that comprises an amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, a fragment thereof, a combination thereof, or a bioactive fragment of said

15   combination.

In another aspect, the invention provides an implantable device having at least one surface or portion of at least one surface that comprises the composition of any one of the above compositions described herein. By way of example, the implantable device may be fashioned as an artificial joint device, such as a knee replacement. The invention may also

20   take the form of a porous vascular graft, wherein at least one region or a portion of at least one region of the porous vascular graft comprises a porous wall that includes the composition of the protein network and covalently attached peptide/protein described herein. The invention as a device may be further defined in other embodiments as a scaffold for skin, bone, nerve or other cell growth, comprising a surface that includes at least one region or area

25   that comprises the composition of the protein matrix and covalently attached peptide described herein.

In yet another aspect, the invention provides for a surgical sealant or adhesive comprising a surface that includes the composition of the peptide matrix and covalently attached peptide on at least one region of the surface.

The invention further provides methods for promoting cell growth or tissue regeneration. This method comprises in some embodiments, covalently attaching or producing a covalently attached bioactive complex molecule comprising a bioactive factor and a transglutaminase substrate, covalently coupling the bioactive complex molecule to a

5   peptide network capable of having covalently attached thereto the bioactive factor or a fragment thereof, to provide a treated peptide substrate; and exposing said treated peptide substrate to a composition comprising cells or tissue to promote cell growth or tissue regeneration. This method may be used in conjunction with a variety of different cell types and tissue types. By way of example, such cell types include nerve cells, skin cells, and bone

10   cells. The peptide network may be further defined as a protein network, such as a fibrin network. The transglutaminase substrate may be further defined as a factor XIII a substrate, while the transglutaminase may be further defined as factor XIIIa. The factor XIII a substrate may be further defined as having an amino acid sequence of SEQ ID NO. 12, SEQ ID NO. 13, SEQ ID NO. 14, SEQ ID NO. 15, a fragment thereof, a combination thereof, or a

15   bioactive peptide fragment of said composition. The peptide may, in some embodiments, be further defined as comprising an amino acid sequence of SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, SEQ ID NO. 6, a fragment thereof, a combination thereof or a bioactive peptide fragment thereof.

The invention in yet another aspect may be defined as a biosupportive matrix

20   material. This material in some aspects, comprises a peptide network and a bioactive factor, wherein said bioactive factor is covalently attached to the peptide substrate. This peptide substrate may be further defined as a protein network. The bioactive factor is covalently attached to the substrate through a transglutaminase or a similar enzyme. The peptide that may be used in conjunction with the invention may comprise any variety of peptides capable

25   of being covalently attached to the fibrin substrate or biosupportive matrix as described herein. In some embodiments, the peptide may be further defined as comprising an amino acid sequence of SEQ ID NO. 1, SEQ ID NO. 4, SEQ ID NO. 5, SEQ ID NO. 10, a fragment thereof, a combination thereof, or a bioactive fragment thereof.

In particular embodiments of the matrix compositions, the calculated moles of peptide

30   that is to be included may be defined or described for those devices/surfaces that include them, as virtually any amount of peptide that falls within a physiologically relevant concentration of the particular peptide/protein selected. For a standard gel, 1 mg of fibrinogen would typically be included. Hence the concentration of fibrinogen in this

standard gel may be described as about 3×10^{-6} mM. Using this figure as a benchmark in one example, the ratio of the amount of peptide to fibrinogen could be expressed as about 3×10^{-6} mM to about 24×10^{-6} mM.

5   **BRIEF DESCRIPTION OF THE DRAWINGS**

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed 10 description of specific embodiments presented herein.

FIG 1. The homodimeric structure of fibrinogen has been elucidated. Each symmetric half of fibrinogen is itself a heterotrimer of the three chains Am, BE and y. Here, the cleavage sites of the major proteases have been marked (*R* is for reptilase, *T* is for thrombin and *P III* is for protease III). Additionally, some of the sites where cross linking 15 can occur have been marked as xl.

FIG 2. A schematic representation of fibrinogen is given. The polymer is held together by the binding of sites B to B' and A to A'. A' and B only become available for binding after cleavage by a protease. The polymerization reaction is self-activated. A single monomer unit is boxed in the center.

20   FIG 3. Each curve represents the different crosslinking abilities of the four peptides. The molar excess of peptide used is plotted against the ratio of peptide molecules to fibrinogen molecules for a series of peptide concentrations. Gln and Lys represent the two peptides that mimic the g-chain of fibrinogen. Polylys is the multiple lysine peptide and pi-1 is the sequence from α2-plasmin inhibitor.

25   FIG 4. Each curve represents the different crosslinking abilities of the four peptides. The molar ratio of peptide to fibrinogen in the initial reaction mixture is varied and plotted on the *x* axis. The ratio of crosslinked peptide to fibrinogen is then measured and plotted on the *y* axis.

FIG 5. Growth normalized against unmodiied fibrin.

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DETAILED DESCRIPTION
OF THE PREFERRED EMBODIMENTS

Following long-standing patent law convention, the terms 'a" and "an" mean "one or
5   more" when used in this application, including the claims.

Using standard solid phase peptide synthesis, peptides with sequences that combine
crosslinking sites from fibrinogen or another protein that crosslinks to fibrin gels, and active
sequences, such as RGD or IKVAV (SEQ ID NO: 4) were created. A dansyl group was
added to the primary amine of the peptide so that the molecule could be detected when in the
10   presence of other proteins. The peptides were syringe filtered and freeze dried to purify.

Fibrin gels were created using thrombin as the enzyme. Thrombin, calcium,
dansylated peptide and Tris Buffered Saline (pH 7) were mixed to achieve the proper
concentration of all components. Dialyzed fibrinogen that contains residual factor XIII was
added and the gels were polymerized in an incubator. The final gel concentrations for each
15   component were 4 mg/ml of fibrinogen, 2.5 mM CA^{++}, 2 NIH units/ml of thrombin and
various amounts of peptide. The gels were then covered with Phosphate Buffered Saline, and
the buffer was changed until all the free peptide had diffused from the gel. The gels were
then degraded with the minimal amount of plasmin necessary to achieve complete
degradation.

20   One method used to analyze the results is as follows. The resulting products were run
out on a gel permeation chromatography column and analyzed using a photodiode array
detector. With this detector, we can collect and analyze data at many wavelengths at the
same time. Chromatograms of each run were made at 280 nm (this signal is proportional to
the total protein present. 205 rim can be used as well). The results were compared to a
25   standard curve created from degraded fibrinogen and the total fibrin concentration was
calculated. A fluorescence detector was used to measure the presence of peptide. The
sample was excited at a wavelength of 330 nm and the emitted energy at 530 rim was
measured (this is proportional to the total amount of dansyl groups present). These results
were compared to standards curves created for each peptide and the ratio of peptide
30   molecules to fibrin molecules in the gel was determined for a series of peptide
concentrations. Furthermore, since a size exclusion column was used, it could be determined
if the size of the peptide fragments in the gel were larger, smaller or the same as that of free

peptide. If they are larger, then this is evidence that the peptide is directly bound to some fragment of gel and a covalent bond has actually been formed.

A second method used to analyze the substrates of the present invention for amount of peptide was as follows. Each gel was washed several times, and the amount of peptide

5   present in each wash was measured on a spectofluorimeter. The gels were then degraded with plasmin and then the amount of fluor present was measured. The percent of fluor in the gel compared to the washes was calculated and since the initial peptide mass is known, the mass of peptide in the gels was calculated from this. When the fibrinogen was dissolved, the total mass dissolved was known and this was used to determine the mass of fibrinogen

10   present in the gel. A different concentration of peptide was used in each series of studies and curves relating the total peptide incorporated with the initial peptide used were made.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function

15   well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

20

EXAMPLE 1
PEPTIDE BOUND PER MOLECULE OF
FIBRINOGEN TO FIBRIN GELS

25   By washing peptide decorated gels, degrading them with plasmin and performing size exclusion chromatography, the amount of peptide bound per molecule of fibrinogen was calculated for a series of peptide concentrations and for four separate peptide sequences. All the substrate sequences tested included RGD as an exemplary bioactive sequence. The sequences tested include two that mimic the crosslinking site in the δ chain of fibrinogen,

30   *YRGD*TIGEGQQHHLGG* (SEQ ID NO: 8) (* indicates the dansyl group and the section in italics is the native sequence of the crosslinking region of fibrinogen), a peptide with glutamine at the transglutaminase coupling site, and *LRGD*GAKDV* (SEQ ID NO: 9), a mimic of the lysine coupling site. Additionally a peptide with a polylysine at a random

-12-

coupling site, *LRGDKKKKG (SEQ ID NO: 10), and a sequence that mimics the crosslinking site in α 2-plasmin inhibitor, *LNQEQVSPLRGD (SEQ ID NO: 11) were also used. The amount of peptide covalently bound to the fibrin gels was measured while varying the initial excess of peptide for each of the four sequences. A concentration dependent curve

5   was created (FIG 3) and the maximum crosslinking ratio and the molar excess needed to achieve a 1:1 ratio are shown below in Table 1. Since a particular active sequence is usually present once in each protein, the excess of peptide required to achieve this concentration provides an interesting benchmark. The peptide that provides the greatest possible crosslinking concentration will provide the most flexibility. From the results seen in FIG 4,

10 the plasmin inhibitor peptide is the best, since it provides the highest crosslinking concentration and the greatest crosslinking efficiency.

TABLE 1

Peptide Sequence	Maximum Crosslinking Ratio Pep/Fibrinogen	Molar excess needed to achieve 1:1 ratio
*YRGD*TIGEGQQHHLGG* SEQ ID NO: 8	1.53	12
*LRGD*GAKDV* SEQ ID NO: 9	0.44	>330
*LRGDKKKKG SEQ ID NO: 10	1.2	11
*L*NQEQVSPLR*GD SEQ ID NO: 11	8.2	6

25 This table shows the amount of peptide needed to covalently bind one peptide molecule per fibrinogen molecule in a fibrin gel.

A collection of peptides utilizing the crosslinking sequence from α2-plasmin inhibitor have been made using active peptide sequences from the basement membrane molecules laminin and collagen SEQ ID NO: 11, and 16-20). Eight day chicken dorsal root ganglia

30 were polymerized inside gels that had enough peptide to achieve the highest crosslinked concentration possible (8 moles peptide/mole fibrinogen). The extension of neurites from the ganglia was measured at 24 and 48 hours. The 48 hour data is shown in FIG 5. The

average neurite length for each experimental condition was normalized against growth in unmodified fibrin. Four of the active peptides used, IKVAV (SEQ ID NO: 4), RNIAEIIKDI (SEQ ID NO: 5), YIGSR (SEQ ID NO: 1) and RGD demonstrated statistically different neurine growth, proving that not only can different factors be attached to the fibrin gels, but

5   they retain biologically significant activity. Soluble inhibitor experiments were completed as well, and in each trial, the neurite growth was statistically the same as unmodified fibrin. This result demonstrates that the activity is interrupted, then the presence of crosslinked peptide does not inhibit neural extension. the growth in RDG crosslinked fibrin also supports this conclusion, as the neurites are able to attain similar growth with this nonactive peptide

10   presence as is achieved in unmodified fibrin.

EXAMPLE 2

The present example is provided to demonsrate the utility of the present invention for

15   provising the covalent attachment of a bioactive factor to a peptide matrix, the amount of the bioactive factor, such as a peptide, being quantitatively determinable.

Using the spectrofluorimetry method (second method) described above, the amount of peptide bound per molecule of fibrinogen was calculated for a series of peptide concentrations and for four separate peptide sequences. The sequences tested include two

20   that mimic the crosslinking site in the γ chain of fibrinogen, *YRGD*TIGEGQQHHLGG* (SEQ ID NO: 8) (* indicates the dansyl group and the section in italics is the native sequence of the crosslinking region of fibrinogen), a peptide with glutamine at the transglutaminase coupling site, and *LRGD*GAKDV* (SEQ ID NO: 9), a mimic of the lysine coupling site. Additionally a peptide with a polylysine at a random coupling site, *LRGDKKKKG (SEQ

25   ID NO: 10), and a sequence that mimics the crosslinking site in α2-plasmin inhibitor, *L*NQEQVSPL*RGD (SEQ ID NO: 11) were also tested. The coupling of each peptide used was measured by determining the excess moles of peptide needed to get one peptide covalently bound to each fibrinogen molecule present. Since a particular active sequence is usually present once in each protein, this is a suitable benchmark. From the results seen in

30   Figure 3, it is clear that the plasmin inhibitor peptide (pi-1) is the best, the peptide with the sequence of multiple lysines (polylys) has the second highest coupling rate, while the two γ

-14-

chain peptides (gln and lys) follow. The actual amount of peptide needed to achieve a 1:1 ratio of peptide to fibrinogen is shown in Table 2.

TABLE 2

5

Peptide sequence	Molar excess needed to achieve 1:1 ratio
*YRGD*TIGEGQQHHLGG*	110
SEQ ID NO: 8	
*LRGD*GAKDV*	220
SEQ ID NO: 9	
LRGDKKKKG	39
SEQ ID NO: 10	
*L*NQEQVSPL*RGD	~10
SEQ ID NO: 11	

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Table 2 shows the amount of peptide needed to covalently bind one peptide molecule per fibrinogen molecule in a fibrin gel.

A Factor XIIIa substrate has been synthetically coupled to a bioactive peptide sought for incorporation into the fibrin matrix, and it is clear that this bioactive factor need not have been a peptide. While not intending to be limited to any particular mechanism of action or theory of operation, any bioactive or biologically or medically useful molecule or macromolecule could be the bioactive factor. Likewise, the coupling between the bioactive factor and the transglutaminase substrate domain could have been performed by recombinant DNA methodology or any other means. For example, a protein growth factor could be incorporated by recombinantly expressing a fusion protein comprising both a transglutaminase substrate domain and the growth factor domain. Furthermore, the transglutaminase substrate domain could be targeted for a translutaminase other than facor XIIIa. Furthermore, a recombinant form of fibrinogen could be used to form the fibrin network. Furthermore, other proteins that transglutaminase recognizes, such as fibronectin for example, could be coupled to the transglutaminase substrate peptide.

There are numerous applications for these fibrin gels that are derivitized with a bioactive factor. Fibrin is a natural matrix found in the body and is utilized in many ways.

Although fibrin does provide a solid support for tissue regeneration and cell ingrowth, there are few active sequences in the monomer that directly enhance these processes. However, other studies have shown that many proteins, including basement membrane proteins such as laminin and growth factors such as basic fibroblast growth factor, have sequences which

5   directly enhance regeneration or migration. Our method allows us to incorporate an active sequence or entire factor into the gels and create gels which possess specific bioactive properties.

The present invention provides the first description of a means by which to effectively incorporate bioactive factors into fibrin, a therapeutically important material in wound

10   healing and tissue engineering have been provided. Hence a previously unaccomplished goal is presented that provides an important therapeutic material.

EXAMPLE 3
BIOACTIVITY *IN SITU* GANGLIA MODEL

15

Bioactivity can be quantified using cell studies based on the 8-day chicken dorsal root ganglia model. With this model, addition of neuronally active sequences to the peptide can be tested for their ability *in vitro* to enhance neurite extension. Ganglia were dissected from eight day old chicken embryos and fibrin gels were polymerized around them. Peptide with

20   different active sequences was crosslinked into these gels and the unbound peptide was washed out by periodically changing the neuronal media on top of the gels. These ganglia then extend neurites in three dimensions and the projection of these neurites can be captured using imaging software. This image can then be used to calculate the average neurite length. Three control experiments were done. Neurites were grown in fibrin gels without any

25   peptide crosslinked, in fibrin gels with a nonactive peptide crosslinked in and in gels with active peptide crosslinked and soluble peptide present in the media as an inhibitor.

EXAMPLE 4
Nerve Regeneration and Scaffold

The present example demonstrates the utility of the present invention as a tissue
5   regenerational supportive material. In addition, the data here demonstrates the utility of the
invention for supporting the effective regeneration of nerve tissue.

A collection of peptides utilizing the crosslinking sequence from α2-plasmin inhibitor
have been made using active peptide sequences from the basement membrane molecules
laminin and collagen. Eight day chicken dorsal root ganglia were polymerized inside gels
10   that had enough peptide to achieve the highest crosslinked concentration possible (8 moles
peptide/mole fibrinogen). The extension of neurites from the ganglia was measured at 24 and
48 hours. the 48 hour data is shown in FIG 5. The average neurite length for each
experimental condition was normalized against growth in unmodified fibrin. Four of the
active peptides used, IKVAV (SEQ ID NO: 4), RNIAEIIKDI (SEQ ID NO: 5), YIGSR (SEQ
15   ID NO: 1) and RGD, demonstrated statistically different neurite growth, proving that not only
can different factors be attached to the fibrin gels, but they retain biologically significant
activity. Soluble inhibitor experiments were completed as well, and in each trial, the neurite
growth was statistically the same as unmodified fibrin. This result demonstrates that the
activity of each sequence added is dependant on the physical crosslinking. Furthermore, this
20   shows that if the neuronal activity of the attached factor is interrupted, then the presence of
crosslinked peptide does not inhibit neural extension. The growth in RDG crosslinked fibrin
also supports this conclusion, as the neurites are able to attain similar growth with this
nonactive peptide present as is achieved in unmodified fibrin.

All of the compositions and methods disclosed and claimed herein can be made and
25   executed without undue experimentation in light of the present disclosure. While the
compositions and methods of this invention have been described in terms of preferred
embodiments, it will be apparent to those of skill in the art that variations may be applied to
the composition, methods and in the steps or in the sequence of steps of the method described
herein without departing from the concept, spirit and scope of the invention. More
30   specifically, it will be apparent that certain agents which are both chemically and
physiologically related may be substituted for the agents described herein while the same or
similar results would be achieved. All such similar substitutes and modifications apparent

to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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        involved in factor XIIIa fibrin crosslinking," *Journal of Biological Chemistry*,
        271:19288-19297, 1996.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

        (i) APPLICANT: California Institute of Technology
            (A) STREET: 1200 East California Blvd.
                         Mail Code 210-85
            (B) CITY: Pasadena
            (C) STATE: California
            (D) COUNTRY: US
            (E) ZIP: 91125

        (ii) TITLE OF INVENTION: Enzyme-Mediated Modification of
                                   Fibrin for Tissue Engineering

       (iii) NUMBER OF SEQUENCES: 20

        (ix) TELECOMMUNICATION INFORMATION:
             (A) TELEPHONE: (512) 495-8400
             (B) TELEFAX: (512) 495-8612

(2) INFORMATION FOR SEQ ID NO:1:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 5 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS:
            (D) TOPOLOGY: linear

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

        Tyr Ile Gly Ser Arg
        1   5

(2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 5 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS:
            (D) TOPOLOGY: linear

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

        Leu Arg Gly Asp Asn
        1   5

(2) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:

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            (A)  LENGTH: 5 amino acids
            (B)  TYPE: amino acid
            (C)  STRANDEDNESS:
            (D)  TOPOLOGY: linear

5
       (xi)  SEQUENCE DESCRIPTION: SEQ ID NO:3:

        Pro Asp Gly Ser Arg
        1                5
10


    (2)  INFORMATION FOR SEQ ID NO:4:

        (i)  SEQUENCE CHARACTERISTICS:
15          (A)  LENGTH: 5 amino acids
            (B)  TYPE: amino acid
            (C)  STRANDEDNESS:
            (D)  TOPOLOGY: linear


20      (xi)  SEQUENCE DESCRIPTION: SEQ ID NO:4:

        Ile Lys Val Ala Val
        1                5


25
    (2)  INFORMATION FOR SEQ ID NO:5:

        (i)  SEQUENCE CHARACTERISTICS:
            (A)  LENGTH: 10 amino acids
30          (B)  TYPE: amino acid
            (C)  STRANDEDNESS:
            (D)  TOPOLOGY: linear


       (xi)  SEQUENCE DESCRIPTION: SEQ ID NO:5:
35
        Arg Asn Ile Ala Glu Ile Ile Lys Asp Ile
        1                5                   10



40  (2)  INFORMATION FOR SEQ ID NO:6:

        (i)  SEQUENCE CHARACTERISTICS:
            (A)  LENGTH: 4 amino acids
            (B)  TYPE: amino acid
45          (C)  STRANDEDNESS:
            (D)  TOPOLOGY: linear


       (xi)  SEQUENCE DESCRIPTION: SEQ ID NO:6:

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(2) INFORMATION FOR SEQ ID NO:7:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 6 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS:
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Pro Arg Arg Ala Arg Val
1   5

(2) INFORMATION FOR SEQ ID NO:8:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 16 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS:
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Tyr Arg Gly Asp Thr Ile Gly Glu Gly Gln Gln His His Leu Gly Gly
1   5   10   15

(2) INFORMATION FOR SEQ ID NO:9:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 9 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS:
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Leu Arg Gly Asp Gly Ala Lys Asp Val
1   5

(2) INFORMATION FOR SEQ ID NO:10:

   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 9 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS:
       (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Leu Arg Gly Asp Lys Lys Lys Lys Gly
1   5

(2) INFORMATION FOR SEQ ID NO:11:

   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 12 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS:
       (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Leu Asn Gln Glu Gln Val Ser Pro Leu Arg Gly Asp
1   5   10

(2) INFORMATION FOR SEQ ID NO:12:

   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 16 amino acids
       (B) TYPE: amino acid

```
          (C)  STRANDEDNESS:
          (D)  TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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     1               5               10               15

(2)  INFORMATION FOR SEQ ID NO:13:

     (i)  SEQUENCE CHARACTERISTICS:
          (A)  LENGTH: 5 amino acids
          (B)  TYPE: amino acid
          (C)  STRANDEDNESS:
          (D)  TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

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(2)  INFORMATION FOR SEQ ID NO:14:

     (i)  SEQUENCE CHARACTERISTICS:
          (A)  LENGTH: 4 amino acids
          (B)  TYPE: amino acid
          (C)  STRANDEDNESS:
          (D)  TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

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(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 8 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS:
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Asn Gln Glu Gln Val Ser Pro Leu
1   5

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 15 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS:
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

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1   5   10   15

(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 15 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS:
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

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Leu Asn Gln Glu Gln Val Ser Pro Leu Asp Asp Gly Glu Ala Gly
1               5                   10                  15
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(2) INFORMATION FOR SEQ ID NO:18:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 16 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Leu Asn Gln Glu Gln Val Ser Pro Leu Arg Ala His Ala Val Ser Glu
1               5                   10                  15
```

(2) INFORMATION FOR SEQ ID NO:19:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 17 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

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Leu Asn Gln Glu Gln Val Ser Pro Arg Asp Ile Lys Val Ala Val Asp
1               5                   10                  15
Gly
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(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 19 amino acids
     (B) TYPE: amino acid
     (C) STRANDEDNESS:
     (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Leu Asn Gln Glu Gln Val Ser Pro Arg Asn Ile Ala Glu Ile Ile Lys
1   5   10   15

Asp Ile Arg

CLAIMS

1.   A composition comprising a protein network and a peptide having an amino acid
sequence that comprises a transglutaminase substrate domain and a bioactive factor, wherein
said peptide is covalently bound to said network.

2.   The composition of claim 1 wherein the protein network is fibrin.

3.   The composition of claim 2 wherein the transglutaminase substrate domain is a factor
XIIIa substrate domain.

4.   The composition of claim 3 wherein the factor XIIIa substrate domain comprises an
amino acid sequence SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, or SEQ ID NO: 15,
a fragment thereof, or a combination thereof, or a bioactive fragment of said combination.

5.   The composition of claim 3 wherein the factor XIIIa substrate domain comprises an
amino acid sequence of SEQ ID NO: 15.

6.   The composition of claim 1 wherein the bioactive factor is a peptide.

7.   The composition of any one of claims 1-5 wherein the bioactive factor comprises an
amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ
ID NO: 5, SEQ ID NO: 6, a combination thereof, or a bioactive fragment thereof.

8.   The composition of claims 1-5 wherein the bioactive factor is a protein.

9.   The composition of claims 1-5 wherein the bioactive factor is a polypeptide growth
factor.

10.   An implantable device having at least one surface comprising the composition of any
one of claims 1-5.

11.   The implantable device of claim 10 further defined as an artificial joint device.

12.   A porous vascular graft comprising in at least one region of a porous wall of said porous vascular graft the composition of any one of claims 1-5.

13.   A scaffold for cell growth comprising a surface that comprises at least one region that includes of the composition of any one of claims 1-5.

14.   The scaffold of claim 13 wherein the cell growth is bone cell growth, sking cell growth, or nerve cell growth.

15.   The scaffold of claim 13 further defined as a scaffold for nerve cell growth.

16.   A surgical adhesive or sealant comprising a surface that includes at least one region of the composition of any one of claims 1-5.

17.   A method for promoting cell growth or tissue regeneration comprising:

covalently attaching or producing a covalently attached bioactive factor to a transglutaminase substrate to provide a bioactive complex molecule;

covalently coupling the bioactive complex molecule to a peptide network capable of having covalently attached thereto the bioactive factor, or a fragment thereof, to provide a treated peptide substrate; and

exposing said treated peptide substrate to a composition comprising cells or tissue to promote cell growth or tissue regeneration.

18.   The method of claim 17 wherein the composition comprising cells are nerve cells.

19.   The method of claim 17 wherein the composition comprises skin cells.

-31-

20.   The method of claim 17 wherein the peptide network is a protein network.

21.   The method of claim 20 wherein the protein network is a fibrin network.

5   22.   The method of claim 21 wherein the transglutaminase substrate is a factor XIIIa substrate and the transglutaminase is factor XIIIa.

23.   The method of claim 22 wherein the factor XIIIa substrate comprises an amino acid sequence of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, or a

10   combination thereof, or a bioactive peptide fragment thereof.

24.   The method of claim 23 wherein the factor XIIIa substrate comprises an amino acid sequence of SEQ ID NO: 15.

15   25.   The method of any one of claims 17-24 wherein the bioactive factor is a peptide.

26.   The method of any one of claims 17-24 wherein the bioactive factor is a protein.

27.   The method of any one of claims 17-24 wherein the bioactive factor is a polypeptide

20   growth factor.

28.   The method of any one of claims 17-24 wherein the peptide comprises an amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, a combination thereof, or a bioactive peptide fragment thereof.

25

29.   A biosupportive matrix material comprising a peptide network and a bioactive factor, wherein said bioactive factor is covalently attached to said peptide substrate.

30.   The biosupportive matrix material of claim 29 wherein the peptide network is further

30   defined as a protein network.

31.   The biosupportive matrix material of claim 29 or 30 wherein the bioactive factor is covalently attached to said substrate through a transglutaminase.

32.   The biosupportive material of claim 29 wherein the peptide has an amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 10, a combination thereof, or a bioactive fragment thereof.

33.   The biosupportive material of claim 32 wherein the peptide has an amino acid sequence of SEQ ID NO: 10.
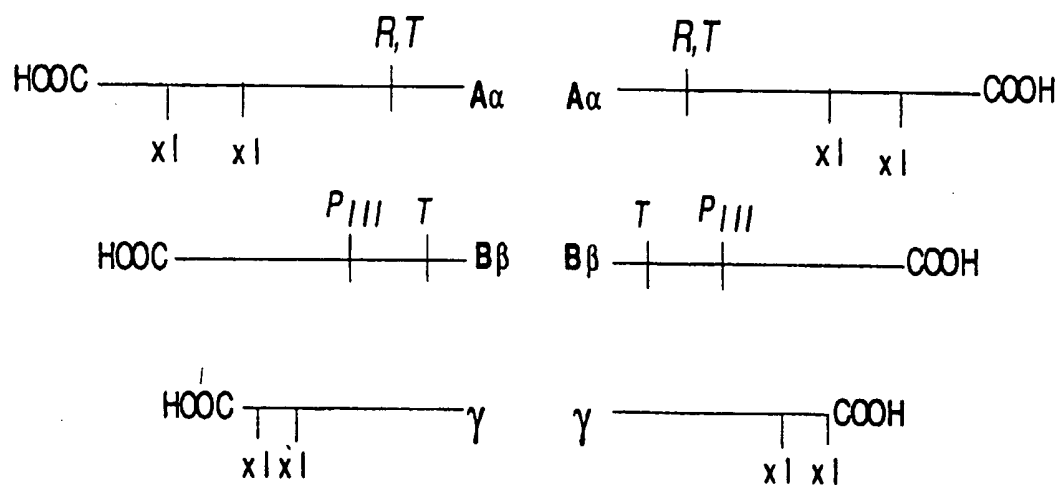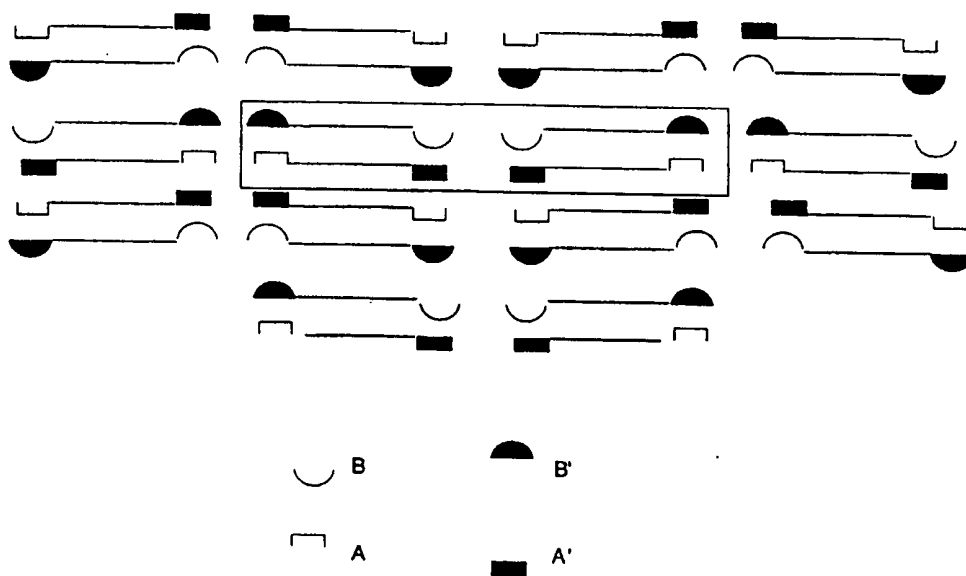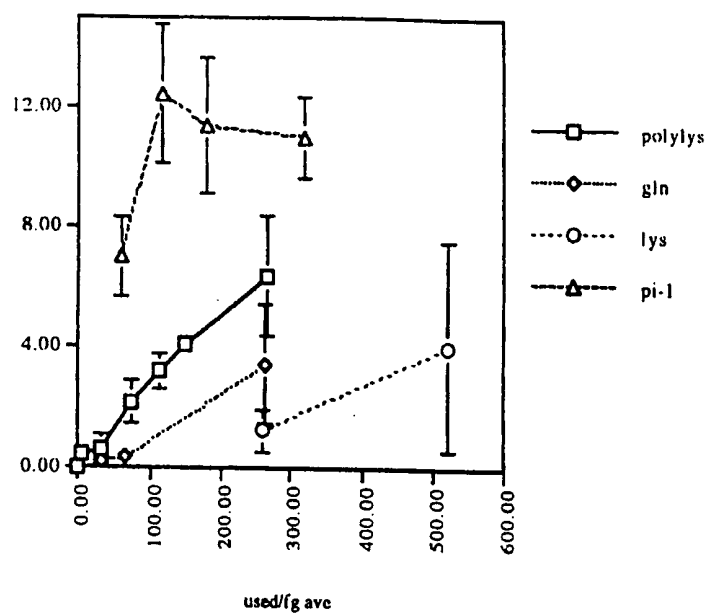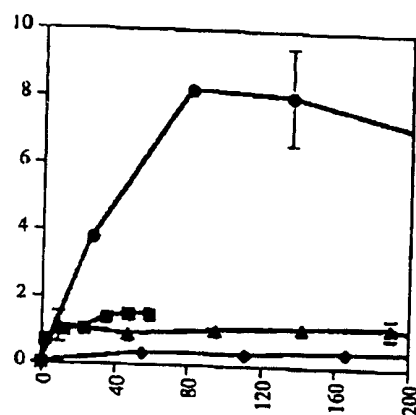
FIG 1

FIG 2

FIG 3

FIG 4
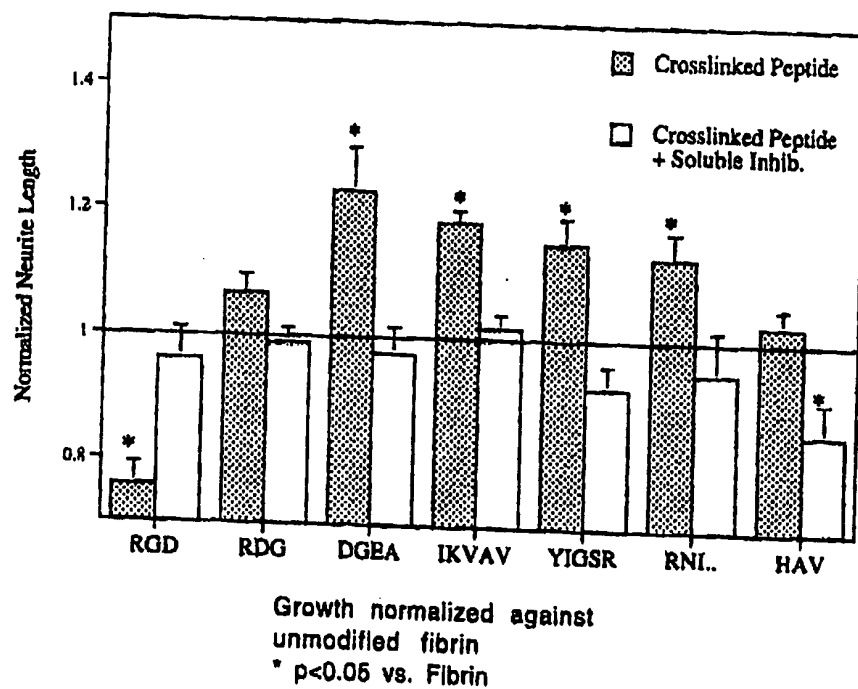
FIG 5

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   A61L25/00   A61L27/00   A61L31/00   C07K14/47

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C07K   A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 23611 A (PROTEIN POLYMER TECH INC) 8 September 1995	1-33
	see page 5, last paragraph	
Y	see the whole document	6,7,9-33
	see page 10	
	see page 24, line 5 - line 20	
	see page 34, line 20 - line 30; example 2	
	see page 76	
	see page 78	

X	WO 89 00051 A (CYTRX BIOPOOL LTD) 12 January 1989	1-5,8
Y	see page 8, line 8 - line 10	6,7,9
	see page 9; claims 19-28	

X	US 5 504 001 A (FOSTER DONALD C) 2 April 1996	1-5,8
Y	see page 3, column 1, line 9 - line 25	6,7,9

	-/--	

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 August 1998	13/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cervigni, S

1

Int   ntional Application No

PC T/US 98/06617

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 05396 A (ZYMOGENETICS INC) 23 February 1995	1,3-6
Y	see claims	10-33

A	WO 90 05177 A (SYNTRO CORP) 17 May 1990	

A	WO 92 22312 A (WADSTROEM JONAS) 23 December 1992	

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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			AU	1979295 A	18-09-1995
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US 5504001	A	02-04-1996	NONE		
WO 9505396	A	23-02-1995	US	5428014 A	27-06-1995
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			AU	4642589 A	28-05-1990
			DE	68928532 D	12-02-1998
			DE	68928532 T	28-05-1998
			DK	163690 A	07-09-1990
			EP	0406357 A	09-01-1991
			JP	3502935 T	04-07-1991
			NO	178625 B	22-01-1996
			US	5723588 A	03-03-1998
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			US	5514581 A	07-05-1996
			US	5773249 A	30-06-1998
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			AU	2149192 A	12-01-1993
			EP	0590015 A	06-04-1994
			JP	6508363 T	22-09-1994
			NO	934592 A	14-02-1994
			US	5631011 A	20-05-1997